PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 11616PC2-MLE	**FOR FURTHER ACTION**	See Form PCT/IPEA/416
International application No. **PCT/AU2004/000752**	International filing date *(day/month/year)* 7 June 2004	Priority date *(day/month/year)* 6 June 2003

International Patent Classification (IPC) or national classification and IPC

Int. Cl. [7] C12N 15/86

Applicant

THE UNIVERSITY OF QUEENSLAND et al

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 3 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. [X] *(sent to the applicant and to the International Bureau)* a total of **6** sheets, as follows:
      - [ ] sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      - [ ] sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.

   b. [ ] *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or table related thereto, in computer readable form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).
4. This report contains indications relating to the following items:
   - [X] Box No. I Basis of the report
   - [ ] Box No. II Priority
   - [ ] Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - [ ] Box No. IV Lack of unity of invention
   - [X] Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - [ ] Box No. VI Certain documents cited
   - [ ] Box No. VII Certain defects in the international application
   - [ ] Box No. VIII Certain observations on the international application

Date of submission of the demand 29 March 2005	Date of completion of the report 29 April 2005
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INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/AU2004/000752

Box No. I Basis of the report

1. With regard to the **language**, this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ This report is based on translations from the original language into the following language which is the language of a translation furnished for the purposes of:

☐ international search (under Rules 12.3 and 23.1 (b))

☐ publication of the international application (under Rule 12.4)

☐ international preliminary examination (under Rules 55.2 and/or 55.3)

2. With regard to the **elements** of the international application, this report is based on (*replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report*):

☐ the international application as originally filed/furnished

☒ the description:

pages **1, 3-4, 6-9, 11-15, 17-40** as originally filed/furnished
pages* **2, 5, 10, 16** received by this Authority on **29 March 2005** with the letter of **29 March 2005**
pages* received by this Authority on with the letter of

☒ the claims:

pages **41** as originally filed/furnished
pages* as amended (together with any statement) under Article 19
pages* **42-43** received by this Authority on **29 March 2005** with the letter of **29 March 2005**
pages* received by this Authority on with the letter of

☒ the drawings:

pages **1/8-8/8** as originally filed/furnished
pages* received by this Authority on with the letter of
pages* received by this Authority on with the letter of

☐ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing.

3. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages
☐ the claims, Nos.
☐ the drawings, sheets/figs
☐ the sequence listing (*specify*):
☐ any table(s) related to the sequence listing (*specify*):

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

☐ the description, pages
☐ the claims, Nos.
☐ the drawings, sheets/figs
☐ the sequence listing (*specify*):
☐ any table(s) related to the sequence listing (*specify*):

* *If item 4 applies, some or all of those sheets may be marked "superseded."*

Box No. V **Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	1-29	YES
	Claims		NO
Inventive step (IS)	Claims	1-29	YES
	Claims		NO
Industrial applicability (IA)	Claims	1-29	YES
	Claims		NO

2. Citations and explanations (Rule 70.7)

The invention the subject of the international application relates to a packaging system for regulatable expression of Flavivirus structural proteins in an animal cell. The system comprises a regulatable promoter operably linked to a nucleotide sequence which encodes a Flavivirus structural protein translation product which comprises each of C, prM and E proteins.

The following document, which is considered the most relevant to the novelty and inventive step of the claims, was first referred to in the corresponding International Search Report:

D1 – *Journal of Virology*, 72(7), 5967-77

D1 relates to a Kunjin virus (KUN) replicon RNA encapsidated by two consecutive electroporations of BHK-21 cells, the first with a KUN replicon RNA C20DXrep that had prME and most of C deleted followed by a recombinant SFV replicon RNA expressing the KUN structural proteins. The prME and the C genes were under the control of separate promoters; hence they were expressed as separate translation products. A 10-fold higher titer of infectious particles was obtained than when two different SFV RNAs expressing the prME gene and the C gene separately were used.

Clearly, the present invention can be considered to fulfil the requirements of novelty because the present invention requires that the C protein, the prM protein and the E protein be expressed as only one protein translation product. This provides the present invention advantages over the prior art, such as a vastly increased titer of VLPs (see specification page 3, 3rd-4th paragraphs).

It was the intention of the authors of D1 to express the entire open reading frame coding for the structural proteins C, prM and E in the SFV replicon. However, despite numerous attempts to carry this out, only a reverse orientation fragment could be inserted.

The present inventors have overcome the problems encountered in the prior art, and have achieved remarkable advantages as a result. Hence, an inventive step for claims 1-29 is also acknowledged.

Industrial applicability is also acknowledged.

Preferably, according to the aforementioned aspects the C, prM, and E structural proteins are of Kunjin virus (KUN) origin.

Preferably, according to the aforementioned aspects the flaviviral replicon is of Kunjin virus, West Nile virus or Dengue virus origin.

In particular embodiments, the flaviviral replicon encodes one or more mutated non-structural proteins.

Throughout this specification, unless otherwise indicated, "comprise", "comprises" and "comprising" are used inclusively rather than exclusively, so that a stated integer or group of integers may include one or more other non-stated integers or groups of integers.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Generation and characterization of stable packaging cell line tetKUNCprME. (A) Schematic representation of the plasmid constructs used for generation of stable packaging cell line tetKUNCprME. pEF-tTA-IRESpuro plasmid was used to generate a first stable BHK cell line, BHK-Tet-Off, continuously expressing the tetracycline transactivator (tTA) from the human elongation factor 1α promoter (pEF-1a). tetKUNCprME, expressing KUN structural genes C, prM, and E (KUN CprME) from tetracycline-inducible CMV promoter (P_{minCMV}) was established by transfection of pTRE2CprME-IRESNeo plasmid DNA into BHK-Tet-Off cells and selection or cells growing in the presence of G418 and puromycin (see text). In uninduced tetKUNCprME cells doxycycline (DOX; a form of tetracycline with higher specific activity) binds to tTA and prevents it from binding to the tetracycline responsive element (TRE) and subsequent activation of CprME mRNA transcription from CMV promoter. To induce expression of KUN CprME genes, DOX is removed from the medium resulting in the release of tTA, its binding to TRE, and activation of CprME mRNA transcription from CMV promoter. tetR – Tet repressor protein; VP16 – Herpes simplex virus VP16 activation domain; IRES – EMCV internal ribosome entry site; puro – puromycin N-acetyl transferase; TRE – Tetracycline-response element; Neo – neomycin resistance gene; SV40 polyA – SV40 transcription terminator/poly(A) signal; β-globin polyA - β-globin transcription terminator/poly(A) signal. (B) Production of secreted E protein and VLPs in induced and uninduced tetKUNCprME cells in the presence and absence of KUN

It will be appreciated that the present invention may therefore have the following broad applications to flavivirus replicon packaging:

(i) an ability to package any flavivirus replicon; and/or

(ii) an ability to express any flavivirus structural proteins necessary and sufficient for flaviviral replicon packaging

As used herein, "*flavivirus*" and "*flaviviral*" refer to members of the genus *Flavivirus* within the family *Flaviviridae*, which contains 65 or more related viral species. Typically, flavivirus are small, enveloped RNA viruses (diameter about 45 nm) with peplomers comprising a single glycoprotein E. Other structural proteins are designated C (core) and M (membrane-like). The single stranded RNA is infectious and typically has a molecular weight of about 4×10^6 with an m7G 'cap' at the 5' end but no poly(A) tract at the 3' end; it functions as the sole messenger. Flaviviruses infect a wide range of vertebrates, and many are transmitted by arthropods such as ticks and mosquitoes, although a separate group of flaviviruses is designated as having no-known-vector (NKV).

Particular, non-limiting examples of flavivirus are West Nile virus, Kunjin virus, Yellow Fever virus, Japanese Encephalitis virus, Dengue virus, Tick-borne encephalitis, Murray Valley encephalitis, Saint Louis encephalitis, Montana Myotis leukoencephalitis virus, Usutu virus, and Alkhurma virus.

The term "*nucleic acid*" as used herein designates single-or double-stranded mRNA, RNA, cRNA, RNA-DNA hybrids and DNA inclusive of cDNA and genomic DNA.

In a preferred form, the packaging construct of the invention is a double-stranded plasmid DNA packaging construct.

By "*protein*" is meant an amino acid polymer. Amino acids may include natural (*i.e* genetically encoded), non-natural, D- and L- amino acids as are well known in the art.

A "*peptide*" is a protein having less than fifty (50) amino acids.

A "*polypeptide*" is a protein having fifty (50) or more amino acids.

According to the present invention, a "*packaging construct*" comprises a regulatable promoter operably linked to one or more nucleotide sequences encoding one or more flaviviral structural proteins.

Suitably, the packaging construct comprises a nucleotide sequence encoding structural proteins C, prM and E.

Accordingly a "*flaviviral expression construct*" is an expression vector into which a heterologous nucleic acid has been inserted so as to be expressible in the form of RNA and/or as an encoded protein.

Said heterologous nucleic acid may encode one or more peptides or polypeptides, or encode a nucleotide sequence substantially identical or substantially complementary to a target sequence.

The heterologous nucleic acid may encode any protein that is expressible in an animal cell.

With this in mind, the flaviviral replicon may be modified, adapted or otherwise engineered to be capable of including said heterologous nucleic acid, typically by the introduction of one or more cloning sites, as for example described in International Publication WO 99/28487.

Introduction of a tetracycline transactivator construct, packaging construct or flavivirus expression construct into an animal host cell may be by any method applicable to animal cells. Such methods include calcium phosphate precipitation, electroporation, delivery by lipofectamine, lipofectin and other lipophilic agents, calcium phosphate precipitation, DEAE-Dextran transfection, microparticle bombardment, microinjection and protoplast fusion.

It will be appreciated from the foregoing that the packaging system of the invention may be used for the expression of proteins in animal cells, preferably mammalian cells.

This may facilitate expression of any eukaryotic protein that requires post-translational processing and/or modification provided by animal cells. Non-limiting examples of such proteins include hormones, growth factors, transcription factors, enzymes, recombinant immunoglobulins or fragments thereof, antigens, immunogens and the like.

In a particular embodiment, VLPs produced according to the present invention may be used to infect appropriate animal cells and thereby facilitate expression of the encoded protein in the cells. Appropriate protein purification techniques may then be used to isolate and purify the expressed protein.

Such a system may exploit animal cells which are capable of expressing high levels of replicon-encoded heterologous protein, such as CHO cells although without limitation thereto.

14. The flaviviral packaging system of Claim 10 wherein the replicon encodes on or more one or more mutated structural proteins.

15. The flaviviral packaging system of Claim 14 wherein the mutated structural protein comprises a mutation selected from the group consisting of:

(i) Leucine residue 250 substituted by Proline in the NS1 nonstructural protein.

(ii) Alanine 30 substituted by Proline in the nonstructural protein NS2A;

(iii) Asparagine 101 substituted by Aspartate in the nonstructural protein NS2A; and

(iv) Proline 270 substituted by Serine in the nonstructural protein NS5.

16. The flaviviral packaging system of Claim 10, wherein the regulatable promoter is tetracycline-repressible.

17. The flaviviral packaging system of Claim 16 wherein the regulatable promoter is a tetracycline repressible CMV promoter.

18. The flaviviral packaging system of Claim 10 wherein the flaviviral expression construct is in RNA form.

19. A packaging cell comprising the flaviviral packaging system of Claim 10.

20. A packaging cell comprising the flaviviral packaging system of Claim 16 and a tetracycline transactivator construct.

21. The packaging cell of Claim 19 or Claim 20, which is a BHK21 cell.

22. A method of producing flavivirus VLPs including the step of:

(i) introducing the packaging construct of Claim 1 into a host cell to thereby produce a packaging cell;

(ii) introducing into said packaging cell a flaviviral expression construct comprising:

(a) a flaviviral replicon;

(b) a heterologous nucleic acid; and

(c) a promoter operably linked to said replicon; and

(iii) inducing production of one or more VLPs by said packaging cell.

23. The method of Claim 22, wherein the flaviviral expression construct is in RNA form.

24. Flaviviral VLPs produced according to the method of Claim 22.

25. An immunotherapeutic composition comprising the VLPs of Claim 24 and a pharmaceutically acceptable carrier diluent or excipient.

26. The immunotherapeutic composition of Claim 25, which is a vaccine.

27. A method of producing a recombinant protein including the step of infecting a host cell with the VLPs of Claim 24, whereby said heterologous nucleic acid encoding said protein is expressed in said host cell.

28. The method of Claim 27, wherein the host cell is a mammalian cell.

29. A method of immunizing an animal including the step of administering the immunotherapeutic composition of Claim 26 to the animal to thereby induce an immune response in the animal.

30. The method of Claim 29, wherein the animal is a mammal.

31. The method of Claim 30, wherein the mammal is a human.

However, packaging of KUN replicon RNA into VLPs is relatively elaborative and time consuming and requires two consecutive transfections, first with KUN replicon RNA and after a 24-36hr delay with the SFV replicon, RNA expressing KUN structural genes (Khromykh, *et al.*, 1998. J Virol. **72** 5967-5977) In addition, the maximum titres of VLPs produced using this system were only about 2 to $5x10^6$ infectious VLPs per ml (Khromykh *et al.*, 1998, *supra*; Varnavski & Khromykh, 1999, Virology. **255** 366-375) which makes large scale VLP manufacture difficult and inefficient.

Flavivirus structural proteins appear to be one of the primary causes of viral cytopathicity and virus-induced apoptosis (Nunes Duarte dos Santos *et al.*, 2000, Virology **274** 292-308). Low cytopathicity of flavivirus replicons compared to the full-length RNA (Khromykh, 2000, *supra*) also demonstrates the major contribution of structural proteins to viral cytopathicity. Although stable cell lines expressing a prM and E cassette from DEN2 and JE viruses have been generated, the expression levels were low when the native prM-E genes were used (Hunt *et al.*, 2001, J. Virol. Methods. **97** 133-149). Inactivation of the furin cleavage site in prM protein to produce immature prM-E particles with low fusogenic activity (Konishi *et al.*, J Virol. **75** 2204-2212), or co-expression of anti-apoptotic bcl-2 gene (Konishi & Fujii, 2002, Vaccine. **20** 1058-1067), was required to establish stable cell lines expressing relatively high amounts of prM-E particles. None of these stable cell lines simultaneously expressed all three flavivirus structural proteins.

Previous attempts by the present inventors to generate a stable cell line continuously expressing all three KUN structural genes under control of separate promoters (expressing C and prM-E separately), resulted in great instability of expression, producing only 10—20% positively expressing cells after a few cell passages. Attempts to use these cell lines to produce KUN replicon VLPs resulted in very low VLP titres

OBJECT OF THE INVENTION

It is therefore an object of the invention to provide a flavivirus packaging system that achieves more efficient and/or higher yield VLP production than prior art packaging systems.